

Pharmaceutical

SCIENCE & TECHNOLOGY today

April 2000

Vol. 3, No. 4

pp. 113–150

<http://pstt.trends.com>



The conformation of biopharmaceuticals such as proteins, nucleic acids, whole cells and vaccines, must be preserved in order to retain their biological activity. For effective product development, the formulation scientist must have a good knowledge of the mechanism of degradation of the biopharmaceutical of interest, and its potential impact on its biological activity, metabolic half-life and immunogenicity. This review focuses on approaches to the formulation of macromolecules into biopharmaceutical products, and provides examples of studies that have been undertaken within the authors' laboratories. **See page 129.** Cover design by Geraldine Woods.

REVIEWS

RESEARCH FOCUS

121

Live bacterial vectors for intranasal delivery of protective antigens

by Camille Loch

129

The formulation of biopharmaceutical products

by Dave A. Parkins and Ulla T. Lashmar

138

Recent technological advances in oral drug delivery – a review

by SriKonda Venkateswara Sastry,
Janaki Ram Nyshadham and Joseph A. Fix

FORTHCOMING ARTICLES

- **Recent advances in the use of isotopes for therapeutic and diagnostic applications**
- **Medical therapeutic nanotechnology via conventional lithography**
- **Quantitative neuroimaging in CNS drug development**
- **Building bridges from process R&D: from customer-supplier relations to partnership**

113 UPDATE

The Clinical Trials Directive – a new regulatory regime for Europe?

by Richard Binns and Bryan Driscoll

114 Computer-integrated drug design

provides new leads for thromboembolic disorders

by Anya M. Hillery

115 Vaccinology: the challenges for non-injected approaches

by David J. Brayden

118 AAPS triggers quest for magic bullet

by Andrew W. Lloyd, A. Christy Hunter and Adrian Smith

MONITOR

146 Progress and profiles: formulation technology, drug delivery

IX DIARY/CLASSIFIED

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Page 1

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Abstracting & Indexing

Current Contents (Agriculture, Biology & Environmental Sciences)

Journal number 02213

Periodicals postage is paid at Rahway, New Jersey and additional entry points. *Pharmaceutical Science & Technology Today* (ISSN 1461-5347) is published monthly, January to December in one volume, by Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington, Oxford, UK OX5 1GB. The annual print only library subscription in the USA is \$1,132.00. Distributed in the USA by Mercury Airfreight International, 365 Blair Road, Avenel, NJ 07001, USA. POSTMASTER: Send address corrections to *Pharmaceutical Science & Technology Today*, Elsevier Science, Customer Support Department, PO Box 945, New York, NY 10159-0945, USA.



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Pharmaceutical Science & Technology Today Vol. 3, 2000, 12 issues per annum. Print includes postage and handling.

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The formulation of biopharmaceutical products

Dave A. Parkins and Ulla T. Lashmar

Biopharmaceutical products represent a diverse group of products that includes proteins, peptides, nucleic acids, whole cells, viral particles and vaccines. The conformation of the macromolecule or cell must be maintained to retain biological activity, and animal models for biological activity and characterization assays are often developed in tandem with initial formulation studies. This presents the formulation scientist with a unique set of challenges when compared to those for small molecules. This review focuses on approaches to the formulation of macromolecules into biopharmaceutical products, and provides examples of studies that have been undertaken within the authors' laboratories.

Dave A. Parkins

Head of BioPharmaceutical
Formulation Sciences
GlaxoWellcome Research and
Development
Ware
UK SG12 0DP
tel: +44 (0)1920 883791
fax: +44 (0)1920 883873
e-mail:
DAP1964@GlaxoWellcome.co.
uk

Ulla T. Lashmar

BioPharmaceutical Formulation
Sciences
GlaxoWellcome Research and
Development
Beckenham
UK BR3 3BS
tel: +44 (0)208 6396578
fax: +44 (0)208 6396332

▼ For effective product development the formulation scientist must have a good understanding of the mechanism of degradation of the macromolecule of interest, and its potential impact on such areas as its biological activity, metabolic half-life and immunogenicity. These mechanisms can, however, be complicated; encompassing many interrelated chemical and physical processes^{1,2}. As degradation may occur during the production, isolation, purification, formulation, storage and delivery of the macromolecule, it is necessary for the formulation scientist to work closely with those developing the bioprocessing and analytical methodologies.

Protein and peptide degradation can be broadly divided into those caused by chemical and physical mechanisms³. There are many chemical mechanisms of degradation; these may include bond formation or cleavage, whereas physical mechanisms involve changes in conformation, adsorption, aggregation and precipitation⁴. Nucleic acids generally require a chemical modification to produce irreversible loss of biological activity, although shear stress, aggre-

gation, precipitation and adsorption⁵ can also cause loss of activity.

Chemical degradation

The mechanisms by which proteins and peptides degrade include deamidation, racemization, hydrolysis, oxidation, and disulphide exchange⁴, with deamidation and oxidation being the most common degradation pathways⁶. For nucleic acids the primary mechanism of degradation is the two-step process of depurination and β -elimination^{5,7}.

Primary structure and stability

Examining the primary structure of a protein or peptide can provide an insight into how the molecule may degrade. However, this will probably only be of real use for peptides and macromolecules when the amino acid sequence is exposed on the outer surface of the macromolecule, and therefore able to undergo chemical degradation.

Disulphide bonds contribute to the formation of tertiary structures and the stabilization of secondary structures⁸; disulphide bond interchange is possible at cysteine-cysteine links, leading to aggregation of the macromolecule. Deamidation is the hydrolysis of the side chain amide on glutamine and asparagine, which may be reduced by formulating at a pharmaceutically relevant pH below neutral pH (Refs 1 and 9). However, isomerization is greatest at low pH for asparagine and glutamine. The side chains of tryptophan, methionine and cysteine and, to a lesser extent, tyrosine and histidine are potential oxidation sites; oxidation of these sites are most significant at neutral and alkaline pH. Methionine in particular is prone to oxidation from atmospheric oxygen^{4,9}. Tryptophan is one of the most photosensitive amino acid residues in proteins¹⁰, whereas a lysine-threonine bond indicates susceptibility to copper-induced cleavage. Hydrolysis

of the peptide bond is possible at asparagine–proline and asparagine–tyrosine linkages, suggesting that these sequences are acid labile¹¹. A summary of amino acids and amino acid sequences, potential reactions and formulation strategies is given in Table 1.

Nucleic acids differ from proteins and peptides by virtue of the fact that they are all chains composed of the same limited number of nucleotides. Even though deamidation is a possible degradation path for deoxyribonucleic acid (DNA), it is probably the chemical integrity of the phosphodiester backbone that is more important⁵. Depurination and β -elimination leading to cleavage of the phosphodiester backbone⁷ is acid catalysed¹², and at alkali pH this reaction occurs at a rate 20-fold lower than at neutral pH (Ref. 2). Long-term stability of a product may therefore be improved by formulating the final product to a pH above seven. If depurination and β -elimination are the only degradative mechanisms occurring, formulation above neutral pH may improve stability⁵. Free radical oxidation is another important source of strand damage⁵, with trace metal ions being able to catalyse many of these oxidative processes¹³. One of the most effective ways of reducing free radical oxidation is by inclusion of antioxidants such as ascorbic acid or glutathione¹⁴. Another potential method is to include chelating agents to remove trace metal ions. Ethylenediaminetetraacetic acid (EDTA) has been recommended for chelation of copper and Desferal, diethylenetriaminepentaacetic acid (DTPA), inos-

itol hexaphosphate and ethylenediaminebis(o-hydroxyphenylacetic acid) for iron chelation^{15,16}.

Techniques to examine chemical degradation

Factors such as pH, ionic strength, buffer composition, other excipients, light, atmospheric oxygen, metal ions and temperature may all affect the mechanism of degradation^{6,17}, and should be evaluated. The formulation scientist should avoid being dependent on any single analytical technique as different techniques can often produce apparently contradictory data. A range of complimentary analytical techniques should therefore be employed to ensure that a broad understanding of the degradation process is obtained.

Bioassays are rarely used in early formulation screening studies of proteins and peptides. This is because they are labour-intensive, relatively insensitive and can be variable. To allow for screening of a large number of samples and to measure changes of only a few percent in any given population, gel electrophoresis, size exclusion chromatography (SEC) looking for chain breaks, and aggregation and other high pressure liquid chromatography (HPLC) methods often provide the basis for stability analysis^{18–20}. Light catalysed degradation and aggregation is routinely assessed in our laboratory using a suntest light cabinet as the light source and SEC–HPLC and iso-electric focusing (IEF) for the analysis. Figure 1 illustrates the difference in light-induced degradation for three monoclonal antibodies. Factors such as pH, ionic strength and buffer type can also be evaluated using SEC–HPLC; Figure 2 illustrates the use of the

Table 1. Amino acids or sequences susceptible to chemical degradation, together with formulation strategies to reduce degradation.

Amino acid or sequence	Mechanism of degradation	Formulation strategy
Cysteine–cysteine	Aggregation	Addition of surfactants, polyalcohols and other excipients
Glutamine, asparagine	Deamidation	pH 3–5
Tryptophan, methionine, cysteine, tyrosine, histidine	Oxidation	pH < 7
Methionine	Oxidation	Protect from oxygen
Tryptophan	Photo decomposition	Protect from light
Lysine–threonine	Copper induced cleavage	Chelating agents
Asparagine–proline, asparagine–tyrosine	Hydrolysis	pH > 7

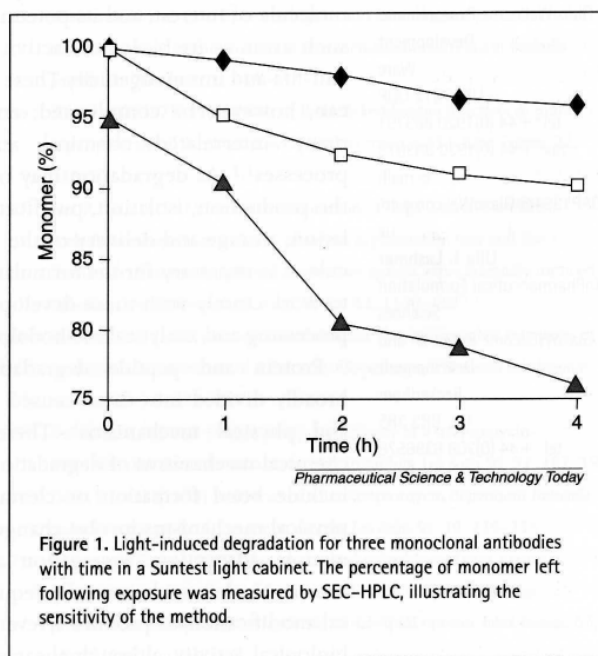


Figure 1. Light-induced degradation for three monoclonal antibodies with time in a Suntest light cabinet. The percentage of monomer left following exposure was measured by SEC–HPLC, illustrating the sensitivity of the method.

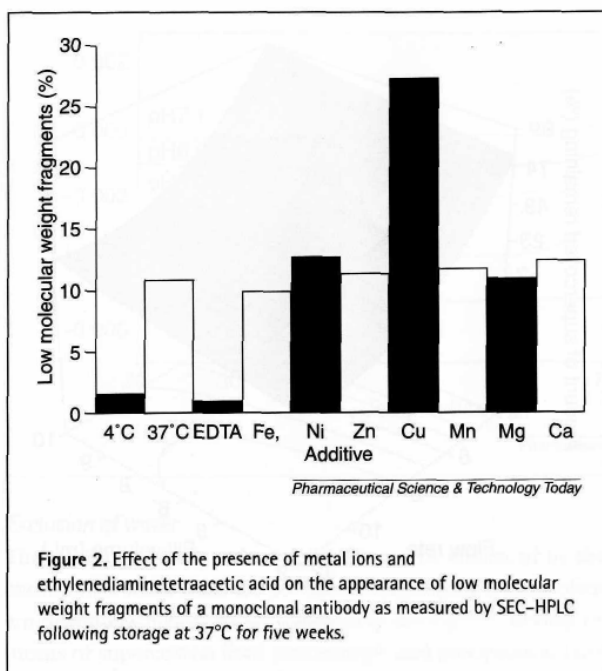


Figure 2. Effect of the presence of metal ions and ethylenediaminetetraacetic acid on the appearance of low molecular weight fragments of a monoclonal antibody as measured by SEC-HPLC following storage at 37°C for five weeks.

technique to evaluate the protective effect of a chelating agent. Circular dichroism (CD) and high sensitivity differential scanning calorimetry (HSDSC) are useful complementary tools for confirming observations made by SEC-HPLC and IEF^{19,21}. DNA formulations are routinely screened using ion-exchange-HPLC, capillary electrophoresis (CE) or agarose gel chromatography. CD and HSDSC can also be used to monitor changes^{5,22-25}.

Physical degradation

Physical instability of proteins involves changes in their secondary, tertiary or quaternary structure and includes denaturation, aggregation, precipitation and surface adsorption. Changes in their conformation also cause loss of potency; it is therefore vital for both the stability and the potency of proteins that conformation is maintained. In contrast, the secondary helix structure for DNA can change without an associated change in potency²⁶. The most common tertiary structure in DNA is supercoiled. A change in the degree of supercoiling or loss of supercoiling to an open circle-form or linear form may not necessarily cause total loss of activity, but may make the nucleic acid more susceptible to aggregation. Therefore ideally, DNA should be formulated in such a manner that its conformation remains relatively unchanged with time⁵.

Aggregation and precipitation

For proteins and peptides aggregation may involve the interchange of covalent bonds, such as disulphide bridges or non-covalent forces such as hydrophobic interactions, and both sol-

uble and insoluble aggregates can exist. Exposure to hydrophobic surfaces, heating, lyophilization, reconstitution of lyophilized material, exposure to light, organic solvents, heavy metals, moisture in solid state and shaking during manufacture, shipping and handling may all induce aggregation^{26,27}, and this may lead to reduced bioactivity²⁸ and immunogenic reactions²⁹. Insoluble aggregates may also lead to unacceptable physical product characteristics such as opalescence and precipitates². While aggregation of most proteins leads to loss of potency, this is not universally true; insulin, for instance, normally exists as dimers or hexamers²⁰.

Techniques to determine aggregation and precipitation

SEC-HPLC is commonly used to determine soluble covalent protein aggregates³⁰. Because the formation of insoluble aggregates involves a change in the number and mass of particles, various techniques, such as light-scattering analysis³¹, spectroscopy^{20,32} and ultracentrifugation³³, have been used to assess the extent of insoluble aggregation.

Fluorescence is a very sensitive technique that will detect insoluble aggregates from protein solutions below 0.01 mg mL⁻¹; this technique is therefore useful as a screening tool for the evaluation of stress-induced aggregation. In our laboratory, fluorescence is used to evaluate the concentration of an excipient required to

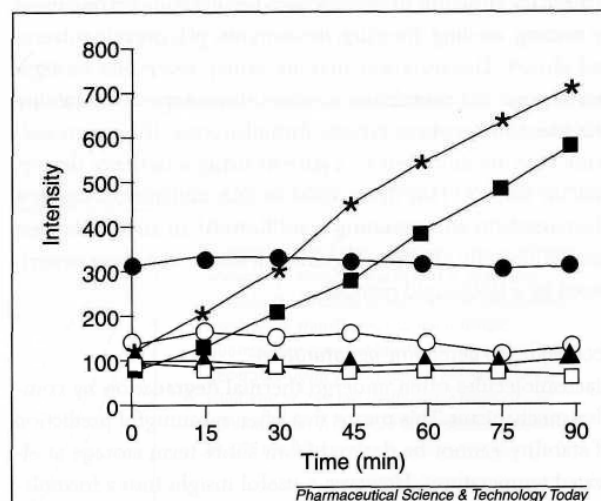


Figure 3. Fluorescence intensity values for 0.1 mg mL⁻¹ antibody in surfactant concentrations from 0% to 0.1%, stirred for up to 90 min at 20°C. The intensity from antibody solutions containing 0.0005% surfactant and above remained constant with stirring time, suggesting that these surfactant concentrations prevent shear-induced aggregation. 0% surfactant (black square); 0.0001% surfactant (star); 0.0005% surfactant (black triangle); 0.001% surfactant (white square); 0.01% surfactant (white circle).

prevent agitation-induced antibody aggregation. Figure 3 illustrates that a surfactant at 0.0005% was sufficient to prevent agitation-induced aggregation in a 0.1 mg mL⁻¹ antibody solution. Electron paramagnetic resonance (EPR) has also been suggested as a tool for the determination of the amount of surfactant required to prevent aggregation³².

Adsorption

Exposure of aqueous solutions of biopharmaceuticals to solid surfaces can lead to loss by adsorption onto liquid-solid interfaces and loss by adsorption. The adsorption of drug to container surfaces or in filters requires particular attention as it can present a significant problem for biopharmaceuticals^{34,35}. Different types of containers and filters have substantially different affinities for macromolecules³⁴. Adsorption is extremely rapid and can be prevented by various additives³⁶.

Techniques to determine adsorption

Loss on contact with a material may be determined by solution depletion using ultraviolet (UV) absorption³⁴, radiolabelling, or other suitable analytical techniques. Desorption and/or exchange techniques have also been used³⁶.

Denaturation

Denaturation refers to an alteration in the secondary, tertiary or quaternary structure of the macromolecule, and can be caused by heating, cooling, freezing, denaturants, pH, organic solvents and shear⁴. Denaturation may be either reversible or irreversible and is a useful tool to assess the comparative stability of a macromolecule in various formulations³⁷. If a macromolecule is to be delivered to a patient using a delivery device, then the impact of the device must be fully understood. Figure 4 illustrates how the operating conditions of an airjet nebulizer can significantly alter the degradation due to the shear experienced by a DNA-lipid complex.

Techniques to determine denaturation

Macromolecules often undergo thermal degradation by complex mechanisms. This means that often meaningful prediction of stability cannot be derived from short-term storage at elevated temperatures. However, a useful insight into a formulation may be obtained by following thermal degradation with the use of calorimetry and determining the impact of formulation variables such as pH, buffer type, ionic concentration and the presence of stabilizing additives^{37,38}. Figure 5 illustrates the variation in onset of denaturation for a monoclonal antibody with varying pH using HSDSC. These events can also be followed by CD and intrinsic fluorescence, as illustrated in Fig. 6. Other techniques of value in the detection of thermal denaturation include UV, nuclear magnetic resonance (NMR),

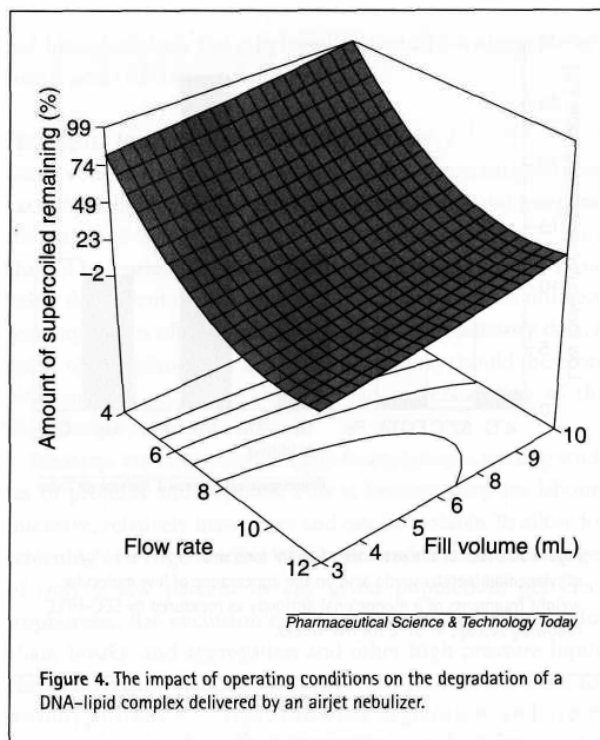


Figure 4. The impact of operating conditions on the degradation of a DNA-lipid complex delivered by an airjet nebulizer.

SEC-HPLC and gel electrophoresis. The study of heat-induced conversion of supercoiled DNA to the open circle and linear forms are typically monitored using agarose gel electrophoresis, capillary electrophoresis and various chromatographic techniques. HSDSC, UV and CD can also be used for screening purposes³⁹.

Formulation strategies for macromolecules

Many macromolecules have a relatively short shelf life in aqueous medium, which can lead to both poor bioavailability and poor stability in the final product. To improve bioavailability the macromolecule may be formulated as an oily injection, sustained release product, particulate delivery system or implant; these types of products often have the advantage of improved stability over an aqueous formulation. Where the formulation does not have adequate stability, then stability might be improved through the exclusion of water from the product or the addition of stabilizing excipients.

When developing a biopharmaceutical product a wide range of formulation components have to be considered; these include buffer type and strength, use of ionic compounds, sugars, polyols and certain amino acids, the incorporation of surfactants, inclusion of antioxidants, chelating agents and other substances. Figure 7 illustrates how a design expert system and denaturation temperature can be used to assist in the selection of several optimum parameters for a liquid formulation of an antibody.

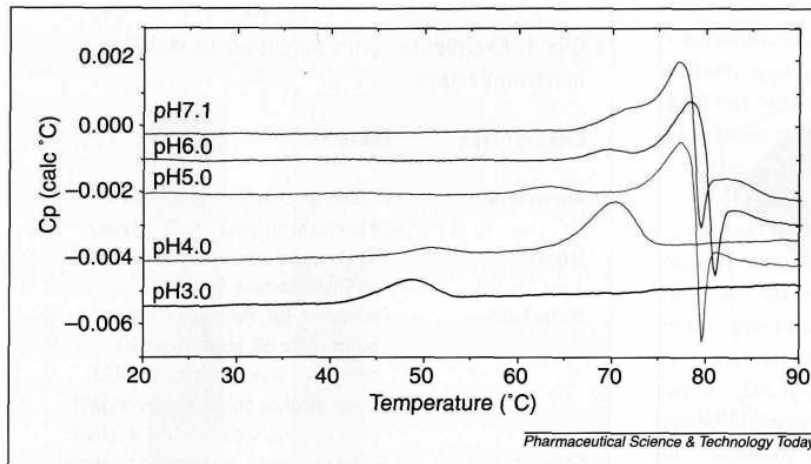


Figure 5. Denaturation profile for a monoclonal antibody at different pH values. Data generated using high sensitivity differential scanning calorimetry.

Exclusion of water

The stability of many macromolecules can be enhanced by the exclusion of water from the product. Freeze-drying is most often employed for this purpose³⁸, but spray drying^{40,41}, drying by means of supercritical fluid processing⁴² and precipitation have also been investigated. Freeze-drying does not always result in a more stable product compared with a solution⁴³. Macromolecules can denature during freezing because of changes in the microenvironment; for instance, from increasing salt concentration or changing pH in the unfrozen liquid^{26,44}. Aggregation and denaturation can also occur during the secondary drying process as a result of the loss of residual water⁴⁵. The rate of freezing has also been found to affect the formation of aggregates⁴⁶. Denaturation during spray drying has been reported as a result of the high air-product interfaces and high

temperatures during drying⁴⁷. The supercritical fluid technique may involve high pressure, high air-product interfaces and possibly solvents, all of which can cause denaturation⁴⁸. Residual moisture in the product can cause both chemical and physical degradation, which, apart from reduced bioactivity, can also cause reduced reconstitution and diffusional properties⁴⁹.

Additives

A variety of excipients can be introduced into a formulation in an effort to increase the stability of the macromolecule during the manufacture, handling and delivery of the product.

Sugars

Sugars have been shown to protect macromolecules against denaturation⁵⁰ and are particularly used as cryoprotectants⁵¹. The

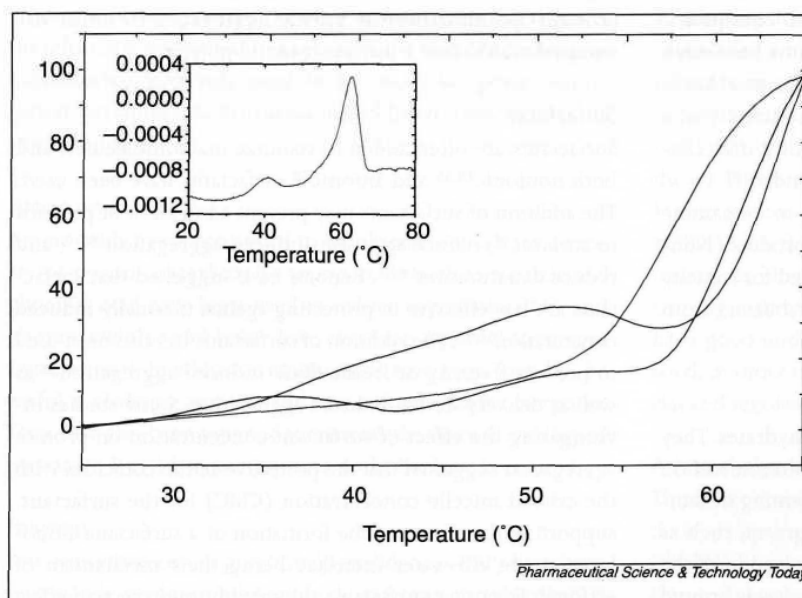
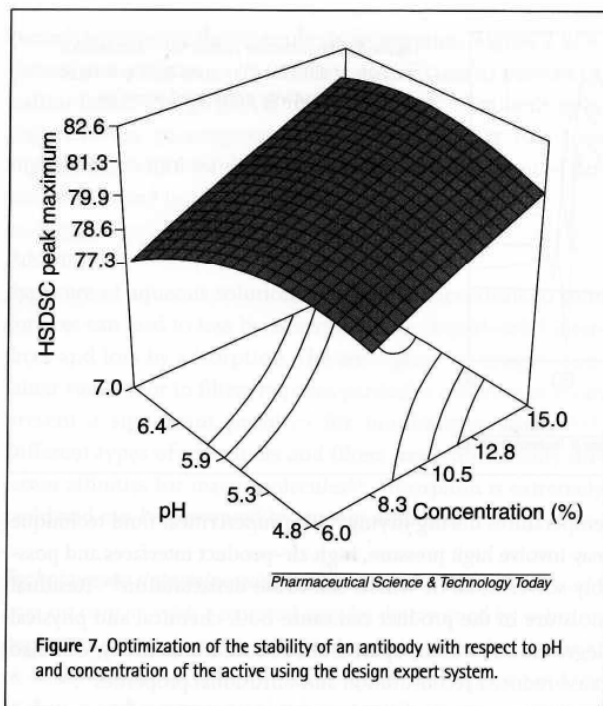


Figure 6. Use of circular dichroism (CD) and fluorescence to follow structural changes. Insert shows high sensitivity differential scanning calorimetry (HSDSC) profile. HSDSC reveals two exothermic transitions. CD and intrinsic fluorescence can also follow these events. Data generated using a monoclonal antibody. Molar ellipticity at 295 nm (red); molar ellipticity at 225 nm (green); intrinsic fluorescence intensity at 350 nm (blue).



mechanism for stabilization is similar in both liquid and frozen systems and has been described by several groups¹⁹. The presence of a sugar creates a thermodynamically unfavourable condition, because the chemical potential – the partial molar free energy for both the macromolecule and the sugar – is increased. Preferential exclusion of the sugar from the surface of the macromolecule minimizes thermodynamic activity, which in turn preserves the preferred conformation^{52,53}. Sugars may, however, not be universally suitable for all conditions. Temperature-dependent hydrophobic interactions between a macromolecule and certain sugars may cause preferential binding at higher temperatures, thus decreasing the activity of a macromolecule¹⁹. For optimum long-term stability, disaccharides such as trehalose and sucrose are recommended¹⁹.

Reducing sugars can convert amide groups to ketoamine groups, resulting in a brown colour of the product. Non-reducing sugars should, therefore, always be used for tonicity adjustments and as stabilizers. A selection of stabilizers from this group are given in Box 1.

Polyols

Polyols include polyhydric alcohols and carbohydrates. They are used to stabilize macromolecules both in solution and on freeze-drying, and are particularly useful in preventing denaturation^{38,45,54,55}. Some of the materials in this group, such as glycerol⁵⁶, stabilize proteins through selective solvation of the protein, causing water molecules to pack more closely around

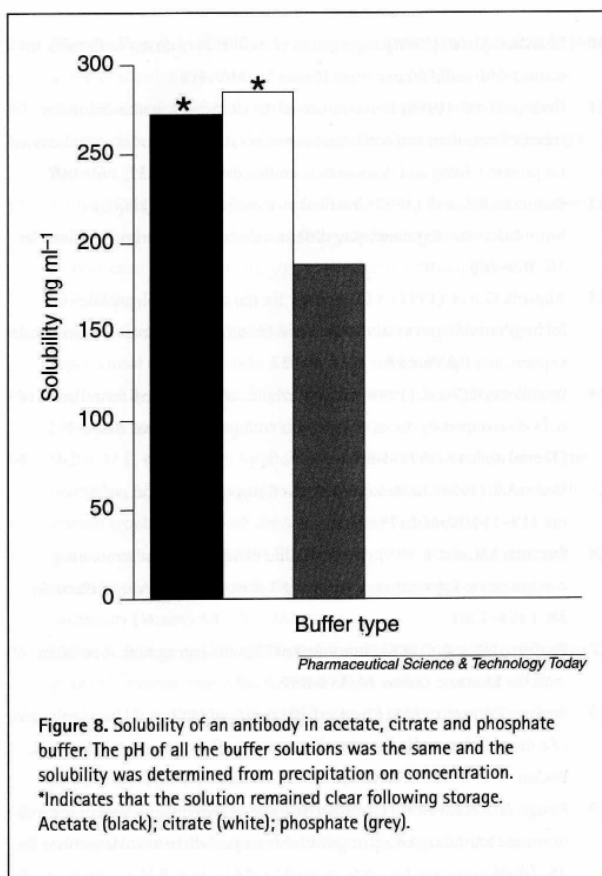
Box 1. Excipients commonly used to stabilize macromolecules

Excipient type	Examples
Amino acids	Glycine, arginine, alanine, proline, aspartic acid, glutamic acid, lysine
Sugars	Trehalose, sucrose, maltose, fructose, raffinose, lactose, glucose
Surfactants	Poloxamer 407, Poloxamer 188, polysorbate 80, polysorbate 20, octoxynol-9, polyoxyethylene-(23) lauryl alcohol, polyoxyethylene-(20) oleyl alcohol, sodium lauryl sulphate
Salts	Sodium sulphate, ammonium sulphate, magnesium sulphate, sodium acetate, sodium lactate, sodium succinate, sodium propionate, potassium phosphate
Polyols	Cyclodextrins, mannitol, sorbitol, glycerol, xylitol, inositol
Antioxidants	Ascorbic acid, glutathione
Polymers	Polyethylene glycol, dextran, polyvinylpyrrolidone
Chelating agents	EDTA, tris(hydroxymethyl)aminomethane (TRIS), diethylenetriaminepentaacetic acid, inositol, hexaphosphate, ethylenediaminebis (O-hydroxyphenylacetic acid), desferal

the protein to exclude the more hydrophobic additive. At higher solvent concentration, the polyols may denature the protein. Cyclodextrins may prevent aggregation by molecular encapsulation⁵⁷. Box 1 lists some useful polyols.

Surfactants

Surfactants are often added to stabilize macromolecules, and both nonionic^{20,55} and anionic⁵⁸ surfactants have been used. The addition of surfactants may prevent adsorption of proteins to surfaces⁵⁹, reduce agitation-induced aggregation^{20,60}, and reduce denaturation^{58,61}. Reports have suggested that surfactants are less effective in protecting against thermally induced denaturation^{52,55}. The addition of surfactants has also been used to prevent freezing or freeze thaw-induced aggregation³⁸ as well as delivery device-induced aggregation. Some studies investigating the effect of surfactant concentration on protein aggregation suggested that the protective action coincides with the critical micelle concentration (CMC) for the surfactant, supporting the theory of the formation of a surfactant monolayer at the air–water interface being their mechanism of action⁶¹. For other surfactants the maximum protective effect



occurred at concentrations much higher than the CMC, indicating that other factors are also involved²⁰. Higher concentrations of surfactants may destabilize a macromolecule^{6,60}. The concentration required to protect a macromolecule should, therefore, be evaluated for each possible encountered stress. Surfactants commonly used to prevent adsorption, aggregation, precipitation, denaturation and freeze-thaw stresses are given in Box 1.

Amino acids

Amino acids have been added to biopharmaceuticals for a variety of reasons; dicarboxylic amino acids such as aspartic and glutamic acid have been used to reduce aggregation^{19,62}, and glycine, arginine and lysine have also been reported to prevent aggregation⁶³. In addition, amino acids have been found to be useful as chelating agents⁶⁴, and may reduce surface adsorption. They have also been reported to increase the thermal stability of proteins⁶⁵. Box 1 lists some amino acids that have been used.

Polymers

Low concentrations of poly(vinylpyrrolidone) (PVP) have been successfully used to inhibit antibody aggregation, whereas PVP

concentrations greater than 1% have been shown to enhance antibody aggregation⁶⁰. Dextran is commonly used in freeze-dried products as a cryostabilizer when it is necessary to raise the collapse temperature of a formulation³⁸. Other excipients from this group are listed in Box 1.

Ionic compounds

Ionic compounds such as salts and buffers interact with macromolecules via non-specific or specific binding⁶⁶. Depending on the type of interaction, a salt may increase thermal stability⁵², increase the solubility and reduce the extent of aggregation⁶⁷. The importance in considering the selection of buffer type is illustrated in Fig. 8. The Figure shows that a 200 mg mL⁻¹ antibody solution required the inclusion of an additional solubilizer if a phosphate buffer was used, whereas the selection of an acetate buffer obviated this need. However, care is required, as proteins do tend to denature in high salt concentrations⁶⁸. Box 1 lists some ionic compounds.

Chelating agents antioxidants and others

Chelating agents and antioxidants are mainly used to prevent chemical degradation and their use as such has already been described in this review^{5,14}. Lipids and fatty acids or their derivatives are used to stabilize proteins and nucleic acids, which is not surprising as many proteins in circulation are known to bind to lipids. Stabilization by fatty acids or their derivatives, in particular phospholipids, results from the association of their polar and non-polar portions with reacting groups on the protein or nucleic acid^{4,69}.

Conclusions

In the past, the formulation of biopharmaceutical products has often been empirical, because products were considered too complex for adequate characterization. Developments in the biotechnology industry have meant that highly purified products, that can be characterized, are increasingly being produced. This is a trend that has been recognized by regulatory bodies such as Center for Biologics Evaluation and Research (CBER) with the introduction of the concept of a 'well characterized' product. A consequence of this change is that it is becoming increasingly important that the formulation scientist has a good understanding of the macromolecule being formulated, in order to produce products that meet patient, commercial and regulatory requirements.

Acknowledgements

The authors would like to acknowledge the contribution of their colleagues in Biopharmaceutical Development at GlaxoWellcome Research and Development, and also Andrew Heron (Department of Chemistry, University of Glasgow, UK).

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